Genisphere®

SIGNAL + SAMPLE AMPLIFICATION PRODUCTS

# Biotin Labeling Kit for 3' Arrays

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## Introduction

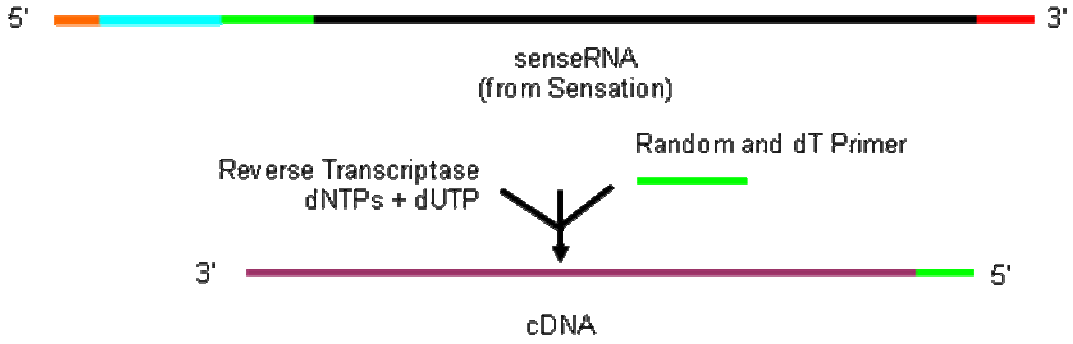
The Biotin Labeling Kit for 3' Arrays contains reagents designed to convert senseRNA from Sensation kits into labeled cDNA, for subsequent analysis on Affymetrix 3' IVT Expression Arrays and other gene expression microarrays. Optionally, unlabeled cDNA may be analyzed by Quantitative PCR (see page 9).

## Biotin Labeling for 3' Arrays

### cDNA Synthesis

*2.5 hours, then 1 hour for purification*

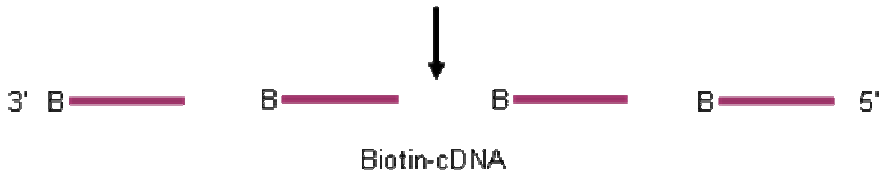
SenseRNA is reverse transcribed using random 12mer primer and dT12V primer, dNTP mix + dUTP, and Reverse Transcriptase. The cDNA is purified with RNAClean XP.




### Terminal Labeling of cDNA

*1 hour*

cDNA is fragmented and end-labeled with 1-Step Fragment and Label Buffer and Enzyme.




### Analysis of Biotin-cDNA

The biotin-cDNA is ready for hybridization to Affymetrix 3' IVT Expression Arrays and other gene expression microarrays.

## Kit Specifications

### Components and Storage

Vial 1 Labeling RT Primer Mix

Vial 2 5X MMLV RT Buffer

Vial 3 MMLV RT Enzyme

Vial 4 RNase Inhibitor

Vial 5 WT dNTP Mix, 10mM dNTP + dUTP

Vial 6 cDNA Stop Solution

Vial 7 1M Tris-HCl, pH 8.0

Vial 8 1-Step 5X Fragment and Label Buffer Mix

Vial 9 1-Step Fragment and Label Enzyme Mix

Vial 10 Nuclease-Free Water

**Store all vials at -20ºC.**

### Handling Kit Components

Vials 1, 2, 6, 7, 10:
Thaw at room temperature, briefly vortex and centrifuge. Keep at room temperature until use.

Vials 3, 4, 9:
Briefly centrifuge, then keep on ice at all times. Do not vortex.

Vials 5, 8:
Thaw on ice, and centrifuge. Keep on ice.

## Additional Materials/Equipment Required

### for Genisphere Biotin Labeling Kit for 3' Arrays

- 10-25μg senseRNA (in a volume of 20μl) from Sensation™ kits from Genisphere
- Thermal Cycler or heating devices for incubations at 4ºC, 25ºC, 37ºC, 42ºC, 47ºC, 65ºC, 80ºC, 93ºC, and 99ºC
- Microcentrifuge
- Agencourt® RNAClean® XP (Beckman Coulter cat. no. A63987)
- Agencourt SPRI®Plate 96R - Ring Magnet Plate (Beckman Coulter cat. no. A29164) or equivalent magnetic plate
- 96-well round bottom microtiter plate (Costar cat. no. 3795)
- 80% Ethanol (Prepare fresh dilutions of ethanol each time RNAClean XP is used)
- 100% Ethanol
- 65ºC heat block or oven for Nuclease-Free Water (Vial 10) during RNAClean Purification
- NanoDrop™ for cDNA quantitation (or equivalent quantitation instrument)
- Optional: Materials for Gel Shift Assay, Appendix A

### for Affymetrix® 3' IVT Expression Arrays

- **SuperScript® II Reverse Transcriptase (Invitrogen™ cat. no. 18064-022)**
- Hybridization oven
- Affymetrix GeneChip Command Console® Software (AGCC)
- Thermal Cycler or heating devices for incubations at 47ºC, 65ºC and 99ºC
- GeneChip® Hybridization, Wash and Stain Kit (Affymetrix cat. no. 900720)
- GeneChip Eukaryotic Hybridization Control Kit (Affymetrix cat. no. 900454)
- Fluidics station
- Scanner

### for Glass Expression Arrays

- Hybridization oven
- Hybridization buffer
- Wash buffers
- Dye-labeled Streptavidin
- Scanner

## Important Parameters

### Choose the Procedure for cDNA Synthesis based on the Application

SuperScript II is recommended for Affymetrix 3' IVT Expression Arrays. Genisphere's Biotin Labeling Kit for 3' Arrays contains MMLV RT enzyme, which is recommended for gene expression microarrays printed on glass.

| Application | RT Enzyme Recommended | Procedure for First Strand cDNA Synthesis |
|---|---|---|
| Affymetrix 3' IVT Expression Arrays | SuperScript II (purchase separately) | Page 6 |
| Gene expression microarrays printed on glass | MMLV (already in kit) | Page 7 |

### Procedural Notes

- 25μg senseRNA (in a volume of 20μl) is recommended for this labeling procedure. If 25μg senseRNA is not available, proceed with 10-25μg senseRNA. For each sample, use as much senseRNA as possible, up to 25μg, and not less than 10μg.
- When preparing master mixes, the enzyme should be added last and just prior to adding the master mix to the reaction. After the master mix or other reagent is added to the reaction, gently tap the bottom of the tube and briefly microfuge.
- A thermal cycler is recommended to prevent evaporation and condensation of the sample. If a thermal cycler is not available, heat blocks or water baths may be used. Microfuge the reactions after incubations, if condensation is observed.

**Thermalcycler Programs:**

| | | Volume |
|---|---|---|
| cDNA Synthesis | | |
| Program 1 | 80ºC - 5 min, 4ºC - 2 min, 25ºC - 3 min | 23μl |
| Program 2 | 42ºC - 120 min, hold at 25ºC for at least 2 min | 40μl |
| Program 3 | 65ºC - 30 min, hold at 25ºC for at least 2 min | 47μl |
| Terminal Labeling of cDNA | | |
| Program 4 | 37ºC - 60 min, 93ºC - 2 min, hold at 4ºC for at least 2 min | 30μl |
| Hybridization Cocktail | | |
| Program 5 | 99ºC - 5 min, 47ºC - 5 min | 140μl or 220μl |

## Procedure for Use

### cDNA Synthesis with SuperScript II Reverse Transcriptase (purchased separately)

This procedure is recommended for Affymetrix 3' IVT Expression Arrays.

25μg senseRNA (in a volume of 20μl) is recommended for this labeling procedure. If 25μg senseRNA is not available, proceed with 10-25μg senseRNA. For each sample, use as much senseRNA as possible, up to 25μg, and not less than 10μg.

1. Adjust the volume of senseRNA to 20μl with Nuclease-Free Water (Vial 10).

2. On ice, add 3μl Labeling RT Primer Mix (Vial 1) to the 20μl senseRNA to make a 23μl senseRNA-Primer Mix.

3. Incubate the 23μl senseRNA-Primer Mix (Thermalcycler Program 1):
   80ºC for 5 minutes
   4ºC for 2 minutes
   25ºC for 3 minutes

4. For each reaction, prepare a First Strand Master Mix in a separate tube on ice:
   8μl 5X First Strand Buffer (from Invitrogen cat. no. 18064-022)
   4μl 0.1M DTT (from Invitrogen cat. no. 18064-022)
   1μl RNase Inhibitor (Vial 4)
   2μl WT dNTP mix, 10mM dNTP + dUTP (Vial 5)
   2μl SuperScript II Reverse Transcriptase (Invitrogen cat. no. 18064-022)
   17μl

5. Add the 17μl First Strand Master Mix to the 23μl senseRNA-primer mix for a volume of 40μl.

6. Incubate the 40μl reactions at 42ºC for 2 hours (Thermalcycler Program 2).

7. Add 7μl of cDNA stop solution (Vial 6) to each sample.

8. Incubate the 47μl samples at 65ºC for 30 minutes (Thermalcycler Program 3).

   Note: during this time, shake the Agencourt RNACleanXP Reagent bottle to resuspend the magnetic particles thay may have settled. Aliquot the appropriate amount of RNACleanXP Reagent, and keep at room temperature. For each reaction,102.6μl will be needed.

9. Add 10μl of 1M Tris-HCl, pH 8.0 (Vial 7) to each sample for a volume of 57μl. Gently mix and briefly microfuge.

10. Proceed to page 8.

**cDNA Synthesis with MMLV (provided in kit)**

This procedure is recommended for gene expression microarrays printed on glass.

25μg senseRNA (in a volume of 20μl) is recommended for this labeling procedure. If 25μg senseRNA is not available, proceed with 10-25μg senseRNA. For each sample, use as much senseRNA as possible, up to 25μg, and not less than 10μg.

1. Adjust the volume of senseRNA to 20μl with Nuclease-Free Water (Vial 10).

2. On ice, add 3μl Labeling RT Primer Mix (Vial 1) to the 20μl senseRNA to make a 23μl senseRNA-Primer Mix.

3. Incubate the 23μl senseRNA-Primer Mix (Thermalcycler Program 1):
   80ºC for 5 minutes
   4ºC for 2 minutes
   25ºC for 3 minutes

4. For each reaction, prepare a First Strand Master Mix in a separate tube on ice:
   8μl 5X MMLV RT Buffer (Vial 2)
   4μl Nuclease-Free Water (Vial 10)
   1μl RNase Inhibitor (Vial 4)
   2μl WT dNTP mix, 10mM dNTP + dUTP (Vial 5)
   2μl MMLV-RT Enzyme (Vial 3)
   17μl

5. Add the 17μl First Strand Master Mix to the 23μl senseRNA-Primer Mix for a volume of 40μl.

6. Incubate the 40μl reactions at 42ºC for 2 hours (Thermalcycler Program 2).

7. Add 7μl of cDNA Stop Solution (Vial 6) to each sample.

8. Incubate the 47μl reactions at 65ºC for 30 minutes (Thermalcycler Program 3).

   Note: during this time, shake the Agencourt RNACleanXP Reagent bottle to resuspend the magnetic particles thay may have settled. Aliquot the appropriate amount of RNACleanXP Reagent, and keep at room temperature. For each reaction,102.6μl will be needed.

9. Add 10μl of 1M Tris-HCl, pH 8.0 (Vial 7) to each reaction for a volume of 57μl. Gently mix and briefly microfuge.

10. Proceed to page 8.

**RNAClean XP Purification**

Note: Prepare fresh dilutions of ethanol each time RNAClean XP is used.

1. If not already done, shake the RNAClean XP Reagent bottle to resuspend the magnetic particles that may have settled. Aliquot the appropriate amount of RNAClean XP Reagent, and bring to room temperature.

2. Place Vial 10, Nuclease-Free Water in a 65ºC heat block or oven.

3. For each reaction, add 102.6µl of RNAClean XP reagent to a well of a Costar 96 well round bottom plate. Transfer the 57µl biotinylated cDNA sample and mix well by pipetting up and down 10-20 times.

4. **Add 86µl of 100% Ethanol to the biotinylated cDNA sample/RNAclean XP mixture and mix well by pipetting up and down 10-20 times. Incubate for 20 minutes at room temperature (20-25°C).**

5. Place the plate onto an Agencourt SPRIPlate 96 Ring magnet (or equivalent) for 10 minutes to separate the beads from the solution.

6. Slowly aspirate and discard the cleared solution, being careful not to disturb the magnetic beads.

7. While on the magnet, add 170µl of **80% ethanol** to each well and incubate for 30 seconds at room temperature. With a pipette set to 200µl, slowly aspirate the ethanol wash solution, being careful not to disturb the magnetic beads. Repeat for a total of 3 washes. **Completely** remove the final wash solution.

8. Air-dry on the magnetic plate for 10 minutes or until all ethanol has evaporated. It may be necessary to extend the air-dry step for an additional 5 minutes until all ethanol has evaporated. Under or over-drying may result in lower sample recovery.

9. Remove the plate from the magnet.

10. Add 27µl of the 65ºC Nuclease-Free Water (Vial 10) to each well. Once water is added to all wells, incubate at room temperature (20-25ºC) for 2-3 minutes to elute the sample from the magnetic beads. During this time, beads can be mixed by gentle shaking of the plate, or by pipetting up and down until resuspended.

11. Place the plate onto the magnetic plate for 2-3 minutes to separate the beads from the solution.

12. Slowly aspirate the purified cDNA sample and transfer to a new tube, being careful not to disturb the magnetic beads. Record the volume recovered.

**Quantitation of cDNA**

Quantitate the cDNA using a NanoDrop or other instrument. From the OD, determine the nucleic acid concentration of each labeled sample:

A260nm x 33 (single-stranded DNA extinction coefficient) x dilution factor
= concentration of cDNA in ng/µl

Calculate the A260/280 ratio to determine purity. A ratio of 1.9-2.1 is desirable.

Optional: Unlabeled cDNA may be analyzed by Quantitative PCR (do not use PCR master mixes containing Uracil N-Glycosylase).

Proceed to terminal labeling with 3-8µg cDNA. **For each sample, label as much cDNA as possible, up to 8µg, and not less than 3µg.** Store remaining cDNA at -20ºC.

**Terminal Labeling of cDNA**

1. Adjust the volume of 3-8µg cDNA to 22.5µl with Nuclease-Free Water (Vial 10). For each sample, label as much cDNA as possible, up to 8µg, and not less than 3µg.

2. For each reaction, prepare a Terminal Labeling reaction master mix in a separate tube on ice:
   6.0µl 1-Step 5X Fragment and Label Buffer Mix (Vial 8)
   1.5µl 1-Step Fragment and Label Enzyme Mix (Vial 9)
   7.5µl

3. Add the 7.5µl Terminal Labeling reaction master mix to the 22.5µl cDNA for a final reaction volume of 30µl.

4. Incubate samples for terminal labeling (Thermalcycler Program 4):
   37ºC for 60 minutes
   93ºC for 2 minutes
   4ºC for 2 minutes

5. Optional: Run the gel shift assay as indicated in Appendix A.

6. Proceed to Affymetrix 3' IVT Expression Array Hybridization (page 10) or Glass Expression Array Hybridization (page 12).

## Affymetrix 3' IVT Expression Array Hybridization

### Preparation of Ovens, Arrays, and Sample Registration Files

1. Turn Affymetrix Hybridization Oven on and set the temperature to **47ºC.** Set the RPM to 60. Turn the rotation on and allow the oven to preheat.

2. Mark each array package with a meaningful designation, and upload the sample and array information (sample names, barcode IDs, etc.) into AGCC.

3. Unwrap the arrays and place on the bench top, and mark each array with the corresponding designation from the wrapper. Allow the arrays to warm to room temperature.

4. Insert a pipet tip into the upper right septum to allow for venting.

### Hybridization

1. Bring the reagents listed in step 3, below, to room temperature. The reagents may be found in the GeneChip Hybridization, Wash and Stain Kit, and the GeneChip Eukaryotic Hybridization Control Kit.

2. Heat the 20X Eukaryotic Hybridization Controls for 5 minutes at 65ºC.

3. For each sample, prepare a Master Mix:

| | **169 format** | **49/64 format** |
|---|---|---|
| Control Oligonucleotide B2 (3nM) | 2.3µl | 3.6µl |
| 20X Eukaryotic Hybridization Controls (BioB, bioC, bioD, cre) | 7µl | 11µl |
| DMSO | 14µl | 22µl |
| Nuclease-Free Water | 16.7µl | 43.4µl |
| 2X Hybridization Mix | 70µl | 110µl |
| **Volume of Master Mix** | **110µl** | **190µl** |

4. Add the Master Mix (step 3) to the biotin-labeled cDNA to prepare the Hybridization Cocktail:

| | **169 format** | **49/64 format** |
|---|---|---|
| Volume of Master Mix | 110µl | 190µl |
| Biotin-labeled cDNA | 30µl | 30µl |
| **Volume of Hyb Cocktail** | **140µl** | **220µl** |

5. Incubate the Hybridization Cocktail (Thermalcycler Program 5):
   99ºC for 5 minutes
   47ºC for 5 minutes

6. Load the appropriate amount of Hybridization Cocktail onto each array:

| | 169 format | 49/64 format |
|---|---|---|
| Volume to Load on Array | 130µl | 200µl |

7. Remove the pipet tip from the upper right septum of the array. Cover both septa with 1/2" Tough-Spots to minimize evaporation and/or prevent leaks.

8. Place the arrays into hybridization oven trays. Load the trays into the hybridization oven.

9. Hybridize with rotation at 60rpm for 16-18 hours at **47ºC**.

**Washing and Staining**

For additional information about washing, staining, and scanning, refer to the HWS Kit User Guide and the Affymetrix Command Console User Guide (http://www.affymetrix.com).

1. Remove the arrays from the oven. Remove the Tough-Spots from the arrays.

2. Extract the hybridization cocktail from each array and transfer it to a new tube or well of a 96-well plate in order to save the hybridization cocktail. Store on ice during the procedure, or at –80ºC for long-term storage.

3. Fill each array completely with Array Holding Buffer.

4. Allow the arrays to equilibrate to room temperature before washing and staining.

NOTE: Arrays can be stored in the Array Holding Buffer at 4ºC for up to 3 hours before proceeding with washing and staining. Equilibrate arrays to room temperature before washing and staining.

5. Place vials into sample holders on the fluidics station:
    a. Place one (amber) vial containing 600µl **Stain Cocktail 1** in sample holder 1.
    b. Place one (clear) vial containing 600µl **Stain Cocktail 2** in sample holder 2.
    c. Place one (clear) vial containing 800µl **Array Holding Buffer** in sample holder 3.

6. Wash the arrays according to array type and components used for Hybridization, Wash and Stain. For HWS kits the protocols are:

| | 169 format | 49/64 format |
|---|---|---|
| Fluidics Protocol | FS450_0002 | FS450_0004 |

7. Check for air bubbles. If there are air bubbles, manually fill the array with Array Holding Buffer. If there are no air bubbles, cover both septa with 3/8" Tough-Spots. Inspect the array glass surface for dust and/or other particulates and, if necessary, carefully wipe the surface with a clean lab wipe before scanning.

**Scanning**

The instructions for using the scanner and scanning arrays can be found in the Affymetrix Command Console Software User Manual in Chapter 6 (http://www.affymetrix.com).

## Glass Expression Array Hybridization

1. Hybridize 3-8μg of biotin-cDNA to the array overnight, with an effective hybridization temperature of 55-65ºC. Use the standard hybridization buffer or Genisphere's 2X SDS Hybridization Buffer, catalog number C600V600S25.

2. Wash per standard protocol, or as follows:
   10 minutes with 55-65ºC 2X SSC, 0.2% SDS
   10 minutes with room temperature 2X SSC
   10 minutes with room temperature 0.2X SSC

3. Dry the array with centrifugation or other standard method.

4. Hybridize dye-labeled Streptavidin (or Genisphere's UltraAmp™ Streptavidin Oyster®-550 reagent, catalog number SA0450) to the array for 30-60 minutes, at room temperature or 37ºC.

5. Wash per standard protocol, or as follows:
   5 minutes with room temperature 1X PBS
   5 minutes with room temperature 1X PBS
   5 minutes with room temperature 1X PBS

6. Dry the array with centrifugation or other standard method.

7. Scan the array.

## References


1. Roberts L, *et al*. Identification of methods for use of formalin-fixed, paraffin-embedded tissue samples in RNA expression profiling. Genomics 2009, 94(5):341-8.

2. Pillai R, *et al*. Validation and Reproducibility of a Microarray-Based Gene Expression Test for Tumor Identification in Formalin-Fixed, Paraffin-Embedded Specimens. Journal of Molecular Diagnostics January 2011, Vol. 13, No. 1.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Patents pending.



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## Appendix A: Gel Shift Assay

Materials Required:

NeutrAvidin
4% to 20% TBE gel
Electrophoresis system with power supply
1X TBE running buffer
DNA Ladder
Gel loading dye
SYBR® Gold Nucleic Acid Gel Stain
Transilluminator

1. Dilute 1µl of each terminal labeling reaction:
   3ul Nuclease-Free water
   1µl terminal labeling reaction
   Note: 2µl of this dilution will be used for the gel shift.
   Save the remaining 2µl for repeat gel shift analysis if necessary.

2. Prepare a NeutrAvidin solution of 2 mg/mL following the manufacturer's recommendation.

3. Place a 4% to 20% TBE gel into the gel holder and load system with 1X TBE Buffer.

4. For each sample to be tested, remove two 1µl aliquots of fragmented and biotinylated sample to fresh tubes. Heat the aliquots of samples at 70ºC 2 minutes.

5. Add 5µl of 2 mg/mL NeutrAvidin to one of the two tubes for each sample tested.

6. Mix and incubate at room temperature for 5 minutes.

7. Bring the volume of DNA ladders to 6µl with water.

8. Add 4µl loading dye to all samples and DNA ladders.

9. Carefully load 10µl samples and ladders on gel.

10. Run the gel at 150 volts until the front dye almost reaches the bottom, approximately 1 hour.

11. While the gel is running, prepare 100mL of a 1X solution of SYBR Gold for staining. SYBR Gold may be diluted in 1X TBE running buffer.

12. Break open cartridge and stain the gel in 1X SYBR Gold for 30 minutes.

13. Place the gel on a UV light box and image using the appropriate filter for SYBR Gold.